THE PATHOGENESIS OF AUTOIMMUNITY IN NEW ZEALAND MICE, I. INDUCTION OF ANTINUCLEIC ACID ANTIBODIES BY POLYINOSINIC·POLYCYTIDYLIC ACID

By Alfred D. Steinberg,* Samuel Baron,† and Norman Talal*

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES AND NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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Abstract.—Antibodies to DNA and RNA were induced in young NZB/NZW F_1 (B/W) female mice following multiple injections of the interferon-inducer polyinosinic·polycytidylic acid (poly I·poly C). Despite serum concentrations of interferon adequate to inhibit the C-type murine leukemia viruses, there was an acceleration of the autoimmune disease in these animals. Anti-RNA, but not anti-DNA antibodies, were induced in B/W male mice, as well as in NZB and NZW mice. Anti-RNA antibodies were also found in 50 per cent of female B/W mice who had never received poly I·poly C and in 8 of 24 sera from patients with systemic lupus erythematosus.

These results suggest that double-stranded RNA functions as a potent antigen in New Zealand mice. Naturally occurring nucleic acids (e.g., viruses) probably act as stimuli to a genetically hyperreactive immune system. According to this hypothesis, the unusual feature in this disease is not a unique virus, but rather the unique genetic susceptibility of the B/W (particularly female) host to immunization with nucleic acids. A similar pathogenetic mechanism may be operative in some humans with systemic lupus erythematosus.

NZB and NZB/NZW F_1 (B/W) mice spontaneously develop an autoimmune disorder resembling human systemic lupus erythematosus. The disease is most severe in female B/W mice who, starting at four months of age, synthesize anti-DNA antibodies and precipitate DNA-antibody complexes in the kidney, leading to nephritis and uremia.[1] A similar pathogenesis has been proposed for human lupus nephritis.[2] In comparison, male B/W mice and NZB mice produce anti-DNA antibodies and develop glomerulonephritis much later in life.

NZB and B/W mice are immunologically hyperreactive and fail to develop or maintain immunologic tolerance to certain experimental antigens.[3, 4] They, like many other mouse strains, are carriers of a C-type murine leukemia virus which contains RNA and is antigenically related to the Gross-leukemia virus.[5] An explanation for their autoimmune disorder may lie in the interrelationship of genetic factors responsible for immunologic hyperresponsiveness and virus infections acting as sources of antigen.

To explore this genetic-viral relationship, we undertook to treat B/W mice from conception with frequent injections of polyinosinic·polycytidylic acid (poly I·poly C) in an attempt to ameliorate the disease. This synthetic double-stranded RNA, a potent inducer of interferon, can control a wide range of viral infections[6, 7] and would be expected to inhibit C-type murine leukemia viruses.[8–11] On the other hand, we anticipated a possible worsening of disease with

such therapy, since rabbits immunized with carrier-protein double-stranded RNA complexes in Freund's adjuvant produce antibodies which cross-react with DNA.[12]

We found that the treated New Zealand mice produced antibodies to poly I·poly C, produced antibodies to DNA earlier and in larger amounts, and showed an acceleration of renal disease as compared to control mice.

Methods.—Animals: New Zealand Black (NZB) and New Zealand White (NZW) mice were obtained from colonies maintained at the National Institutes of Health, Bethesda, Maryland. All F_1 hybrids were obtained by NZB female × NZW male matings.

Poly I·poly C: The double-stranded RNA was formed by annealing equimolar amounts of the single-stranded homopolymers—polyinosinic and polycytidylic acid—in 0.15 *M* NaCl.[13] The poly I·poly C was kindly provided by Dr. Hilton B. Levy. It was kept frozen in aliquots at −20°C at a concentration of 1.0–1.6 mg/ml at pH 7.9 in pyrogen-free buffer. It was used within 1 hr of thawing. Dilutions were made in pyrogen-free saline at pH 7.9 for treatment of young animals.

Treatment schedule: NZB and NZW parents were continuously treated with poly I·poly C (100–150 μg) 3 times a week from 4 weeks of age, so that therapy would be initiated *in utero*. Treatment was continued in the B/W F_1 offspring by injections 3 times weekly beginning at birth. The initial dose (8–16 μg) was doubled weekly until a dose of 75–100 μg was reached, after which therapy was continued at that level (3 μg/gm of body weight). All injections were intraperitoneal.

In other experiments, B/W females were started at 4 weeks of age on a program of 75–150 μg of poly I·poly C 3 times a week. Two groups of control mice were used: (1) untreated mice of varying ages and (2) 4-week-old B/W mice who received footpad injections of complete Freund's adjuvant containing bovine gamma globulin.

Antibody assay: Anti-DNA and anti-poly I·poly C antibodies were detected by a modification of the Farr assay[14] using ^{14}C-labeled KB DNA (kindly provided by Dr. Theodore Pincus) or ^{14}C-labeled poly I·poly C (kindly provided by Dr. Hilton B. Levy). Individual sera diluted 1:4 or 1:10 with borate buffer (pH 8.0) were incubated with 40 mμg of ^{14}C-native DNA (25,000 dpm/μg) or 200 mμg of ^{14}C-poly I·poly C (2,000 dpm/μg) at 37°C for 30 min, then at 4°C for 18 hr. An equal volume (0.1 ml) of ammonium sulfate was added to give a final concentration of 50% for the anti-DNA and 35% for the anti-poly I·poly C assay. Poly I·poly C itself was partially insoluble in ammonium sulfate concentrations exceeding 40%. After 1 hr at 0°C, the precipitate and supernatant fractions were separated by centrifugation, suspended in Bray's solution, and assayed for radioactivity in a liquid scintillation counter as previously described.[14] The results were expressed as the percentage of antigen bound by the serum. In this assay, 40% binding is considered indicative of significant antibody activity.[14]

To study inhibition of binding, the sera were first incubated with a 25- to 125-fold excess of various nonradioactive nucleic acids for 30 min prior to the addition of radioactive DNA or poly I·poly C in the above assay system.

Interferon assay: Interferon titers in the serum were determined as the reciprocal of the highest dilution of serum which inhibited the yield of vesicular stomatitis virus in primary mouse embryo culture or the yield of GDVII virus hemagglutinin in mouse L-cell culture by 0.5 $\log_{10}$.[15]

Fluorescence microscopy: Kidneys were frozen immediately in a Dry Ice-alcohol bath prior to cryostat sectioning. The sections were treated separately with fluorescein-conjugated rabbit antisera to γ globulin or β1*C*, a human lupus anti-DNA antiserum, and a rabbit anti-RNA antiserum. A fluorescein-conjugated goat anti-rabbit serum was used to reveal the latter reaction. Antisera were kindly provided by Drs. Richard Asofsky, David Stollar, and Peter H. Schur. Fluorescence microscopy was kindly performed by Dr. Schur.

Results.—Treated mice: B/W female mice treated with poly I·poly C either from conception or from four weeks of age were found to have much larger quantities of anti-DNA antibody at four and one-half months of age than did untreated or Freund's adjuvant-treated controls (Table 1). The only sera that bound greater than 40 per cent DNA at a 1:10 dilution were from mice treated with poly I·poly C.

Of the 30 New Zealand mice (NZB, NZW, B/W) treated with poly I·poly C, 28 had significant amounts of antibody to the double-stranded RNA (Table 2*A*). The earliest antibody was detected in six-week-old B/W females who had received four injections of poly I·poly C. In high-binding sera, precipitating antibody was demonstrated by double diffusion in agar gel and by immune precipitation with ^{14}C-poly I·poly C. Although treated NZB and NZW mice made just as great quantities of anti-RNA antibodies as female B/W mice, they had no increased incidence of anti-DNA antibodies. B/W male mice resembled the NZB

TABLE 1. *Anti-native DNA antibodies in female mice at age 4^1/$_2$ months.*

Group	Mean % DNA Bound ± SEM (Dilution 1:4)	Mean % DNA Bound ± SEM (Dilution 1:10)	No. > 40%/ total no. (dilution 1:10)
Control	37.2 ± 3.2	21.6 ± 2.2	0/18
Treated with Freund's adjuvant	47.2 ± 4.0	26.9 ± 2.8	0/18
Treated with poly I·poly C	75.0 ± 5.0	57.0 ± 4.9	12/16

TABLE 2. *Anti-native DNA and anti-poly I·poly C antibodies in mouse and human sera.*

(A) *Unselected mice in spontaneous and poly I·poly C accelerated disease.*

Strain	Age (months)	Sex	Poly I·poly C treatment	DNA Binding Mean[a]	DNA Binding Number[b]	Poly I·Poly C Binding Mean[a]	Poly I·Poly C Binding Number[b]
NZB	9–17	F	−	27.0	3/14	30.3	3/14
NZB	6–7	F	+	29.9	1/5	61.0	4/5
NZW	7–12	M	−	15.8	0/6	29.2	1/6
NZW	6	M	+	22.1	0/5	71.1	5/5
B/W	5^1/$_2$	F	−	53.5	9/12	35.2	5/12
B/W	4^1/$_2$	F	+	75.0	16/16	71.3	15/16
B/W	5^1/$_2$	M	−	17.3	0/7	17.2	0/7
B/W	4	M	+	23.5	0/4	78.9	4/4

(B) *Sera of untreated mice selected for specific anti-DNA activity.*

Strain	Age (months)	Sex	Range	DNA Binding Mean[a]	DNA Binding Number[b]	Poly I·Poly C Binding Mean[a]	Poly I·Poly C Binding Number[b]
B/W	8^1/$_2$	F	0–40	20.4	0/7	33.4	2/7
B/W	8^1/$_2$	F	41–70	57.4	5/5	74.5	5/5
B/W	8^1/$_2$	F	71–100	89.5	6/6	49.3	3/6
B/W	12–15	M	71–100	79.6	6/6	47.8	4/6

(C) *Human sera.*

	DNA Binding Mean[a]	DNA Binding Number[b]	Poly I·Poly C Binding Mean[a]	Poly I·Poly C Binding Number[b]
Systemic lupus erythematosus	65.9	18/24	31.6	8/24
Normal controls	15.6	0/10	14.3	0/10

[a] Mean percentages antigen bound.
[b] Numerator, number of mice with antigen binding >40%; denominator, number of mice studied.

and NZW in this regard and failed to show the increased levels of anti-DNA present in their female littermates.

Spontaneous disease: Anti-RNA antibodies were also found during the course of spontaneous disease in female NZB and B/W mice who had never received poly I·poly C (Table 2*A*). These sera did not bind ^{14}C-polyuridylic acid. None of eight sera from one-year-old female C3H control mice had anti-RNA antibodies. Selected B/W sera with known anti-DNA activity from female mice $8^1/_2$ months old or males 12–15 months old were assayed for anti-poly I·poly C antibodies (Table 2*B*). There was no correlation between anti-DNA and anti-RNA activities. The highest binding of the RNA was associated with the intermediate DNA-binding sera.

Eight of 24 sera from human patients with systemic lupus erythematosus also contained significant titers of anti-poly I·poly C antibodies. Control human sera were negative (Table 2*C*).

Antibody inhibition: A series of differential inhibition experiments were performed with poly I·poly C and DNA, to see if the two antibody activities were independent of each other. With mouse serum, native or heat-denatured calf thymus (*CT*) DNA inhibited the binding of ^{14}C-DNA but had little effect on the binding of ^{14}C-poly I·poly C (Table 3). By contrast, poly I·poly C and polycytidylic acid (poly C) inhibited the binding of ^{14}C-poly I·poly C but not the binding of ^{14}C-DNA. Polyinosinic acid (poly I) was less inhibitory than poly C. A human lupus serum behaved similarly in the inhibition studies. These results suggest that the two antibody activities are largely distinct, although a population of cross-reacting antibodies may also be present.

Interferon: Serum interferon levels six hours after the first injection of 100 μg of poly I·poly C were $10^{2.3}$ units/ml in NZW, $10^{3.0}$ units/ml in NZB, and $10^{3.2}$ units/ml in B/W mice. Interferon was measured in sera from B/W female mice treated three times per week for four months with poly I·poly C. Despite the presence of anti-poly I·poly C antibodies, the interferon concentration four hours after 75 μg of poly I·poly C was $10^{2.0}$ units/ml.

Nephritis: The accelerated onset and higher titers of anti-DNA and anti-poly I·poly C antibodies resulted in a more severe kidney disease in B/W female mice. At four months of age, moderately severe glomerulonephritis was present in treated but not control animals. At five months of age, severe proliferative glomerulonephritis with focal and diffuse glomerular hyalinization was

TABLE 3. *Differential inhibition of antigen binding.*

Serum	Inhibitor	Amount (μg)	Antigen Bound (%) DNA	Poly I·poly C
B/W mouse	None	—	71	82
	Native CT DNA	5	20	75
	Denatured CT DNA	5	5	64
	Poly I·poly C	5	70	22
	Poly C	50	80	35
	Poly I	50	—	65
Human lupus	None	—	83	86
	Denatured CT DNA	5	14	76
	Poly I·poly C	5	78	39

found in treated mice. Such a kidney showed glomerular fluorescence with antisera specific for mouse γ globulin, complement, and RNA but not DNA, suggesting the presence of an RNA-containing immune complex fixing complement. A control kidney showed glomerular fluorescence only with the anti-mouse γ globulin serum.

Discussion.—Poly I·poly C has several effects when administered to New Zealand mice: (1) the induction of interferon, (2) the induction of antibodies to poly I·poly C, (3) the earlier induction of antibodies to DNA (in female B/W animals), and (4) the acceleration of nephritis.

The concentration of interferon achieved in the treated mice is adequate to inhibit C-type leukemia viruses and to prevent leukemia.[8-11] The failure of poly I·poly C therapy to prevent or retard anti-DNA formation and nephritis in B/W mice argues against a virus being the primary cause of this disease. Viruses may still play secondary roles as antigens and may influence immunologic reactivity in the New Zealand mice.[3, 4] Moreover, other strains of mice infected with murine leukemia viruses do not develop autoimmune disorders.[16]

The action of poly I·poly C as an antigen is unusual, particularly since the RNA is not complexed to a carrier protein. Almost every treated New Zealand mouse, including the NZB and NZW, produced antibodies to the poly I·poly C. Double-stranded RNAs have adjuvant properties, but this is the first time that an uncomplexed nucleic acid has, by itself, been immunogenic.[17]

The acceleration of nephritis and of antibody formation to native DNA by poly I·poly C is remarkable. We have tried to immunize B/W mice with protein-complexed DNA and have succeeded in producing antibodies to denatured but not to native DNA.[18] The RNA-containing LCM virus will, like poly I·poly C, also accelerate the onset of anti-DNA antibodies and nephritis in B/W mice.[19] We conclude that poly I·poly C mimics the action of viruses in these mice. The explanation for this effect probably resides in the genetically controlled sensitivity of the host to nucleic acid antigens. Double-stranded RNAs such as LCM virus or poly I·poly C probably act either as primary initiators or as secondary "boosters" for the synthesis of antinucleic acid antibodies, depending upon the age and immunologic status of the recipients. The immunologic response to several other antigens is also under genetic regulation.[20]

This regulation is also illustrated by the striking sex difference in anti-DNA formation (both naturally occurring and poly I·poly C-induced) in B/W mice. The female B/W, whose spontaneous anti-DNA antibody appears six months earlier than that of the male, also shows an earlier induction of anti-DNA antibodies by the double-stranded RNA. The sex difference in spontaneous anti-DNA formation by B/W mice is not altered by castration and treatment with opposite sex hormones.[18] Thus, direct genetic factors appear to determine whether antibodies to RNA, DNA, or both will be produced after immunization with nucleic acid.

Our experiments with poly I·poly C suggest that RNA antigens also play a role in the spontaneous autoimmune disorder that develops in these mice. Half the female B/W mice have antibodies that bind poly I·poly C, even though they have never received this RNA. These antibodies are not inhibited by DNA and

probably are different from the anti-DNA antibodies. It is likely that this poly I·poly C binding activity represents a cross-reaction with antibody produced to some other RNA, since antinucleic acid antibodies are known to cross-react widely. These naturally occurring anti-RNA antibodies could be induced by RNA viruses latent or acutely infecting the B/W mice, such as the murine leukemia virus. According to this hypothesis, the unusual feature in this disease is not a unique virus but rather the unique genetic susceptibility of the B/W (particularly female) host to nucleic acid antigens.

There is evidence from family studies that human systemic lupus erythematosus may also be a genetic disorder.[21] The disease is quite heterogeneous in man, and many different antigenic stimuli, including certain drugs, probably lead to anti-DNA and other autoantibody formation in genetically susceptible hosts. The finding of anti-poly I·poly C antibodies in humans with this disorder suggests that RNA containing viruses may function in some patients, as in the B/W mice, to provoke antinucleic acid antibodies and lupus nephritis. A similar observation that certain patients with systemic lupus erythematosus have precipitating antibodies to poly I·poly C and poly A·poly U has recently been made in another laboratory.[22]

* National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014.

† National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014.

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